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The severity of cortical Alzheimer's type changes is positively correlated with increased amyloid-β Levels: Resolubilization of amyloid-β with transition metal ion chelators

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The most consistent diagnostic neuropathological lesion in Alzheimer's disease (AD) is the senile plaque of which the 4 kD amyloid-β (Aβ) peptide is the major proteinaceous component. In this study cortical Aβ levels were immunochemically measured in 70 post-mortem human brains and compared against their neuropathological grading as determined by the densities of amyloid plaques and neurofibrillary tangles. The mean concentration of cortical Aβ/mg protein increased with the severity of the cortical degenerative changes (AD0 < AD1 < AD2 < AD3). Brains with the severe degenerative changes (AD3), corresponded to definite AD cases and exhibited significantly increased concentrations of Aβ (11.1 + 3.08 ng/mg total protein, $n = 17$) when compared with control brains without any degenerative changes (AD0; 0.06 ± 0.06 ng/mg total protein, $n = 14$, $P = 0.003$). The extraction of Aβ from the cortex of AD3 brains was significantly enhanced in a dose dependent manner by the presence of the metal ion chelator N,N,N',N'-tetrakis(2-pyridylmethyl) ethylenediamine (5 mM TPEN, $P < 0.0001$). The chelator/antioxidant 1,2-dithiolane-3-pentanoic acid (lipoic acid), also resolubilized Aβ in a dose-dependant manner. Both chelators also enhanced the extraction of Aβ from the frontal cortex of AβPP-transgenic mice suggesting this animal model of amyloidosis may be useful for evaluating the biochemical and therapeutic effects of chelators/antioxidants on Aβ deposition. In summary our results indicate that increased Aβ load is correlated with the severity of the cortical AD-type changes and that chelators/antioxidants may be useful in reducing neuronal amyloid burden.

1. Introduction

A defining neuropathological feature of the Alzheimer's disease (AD) affected brain is the presence of neuritic plaques and neurofibrillary tangles (NFT) in the hippocampus and neocortex of the brain [14,15, 26,34]. The major protein component of AD neuritic plaques is the small 40–43 amino acid peptide amyloid-β (Aβ) [15,26]. In early-onset familial AD, mutations in the amyloid β-protein precursor (AβPP) or presenilin (PS1 or PS2) genes increase the production of the longer hyperaggregable form of Aβ (Aβ_{1-42}) [5,10,23, 35,48]. In contrast, the more common, sporadic form of AD is not usually associated with such mutations and the predominant form of Aβ is the shorter Aβ_{1-40} form [35]. In these sporadic cases other factors such as pathological chaperones may play a major role in the deposition of Aβ [32,33,45–47,49].

While the density of NFT's has been shown to correlate well with the degree of dementia, they lack specificity for AD as they also are found in other neurological diseases [14,27]. Unlike NFT's, amyloid plaques do not correlate well with the degree of dementia. This is not surprising since amyloid plaques are heterogeneous [19,22,30] and do not account for the total amount of Aβ in the brain. While a causal link between Aβ and the clinical presentation of AD associ-

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ated dementia has not been proven, a recent study has provided evidence that Aβ levels increase in the frontal cortex prior to the formation of NFT's and the onset of dementia [34].

Recently, much attention has focused on the role of metal ions, particularly copper, iron and zinc, and pH in promoting the deposition of Aβ [3,4,7,8,18,25,32] since the concentrations of these metal ions is greatly elevated in the neuropil and plaque deposits of AD affected brain compared with control brain [25]. Aβ has been shown to be rapidly precipitated by physiological concentrations of these metal ions in vitro, and precipitation of Aβ by copper and iron is greatly enhanced by mildly acidic conditions that are representative of physiological acidosis.

Interestingly, precipitation of Aβ by copper and zinc in vitro has been shown to be reversible with chelation [3,18], while the precipitation of Aβ by copper under mildly acidic conditions is pH-reversible [3], suggesting a physiological role for metal ion binding to Aβ [3]. Subsequently, Cherny et al. [9] demonstrated that various transition metal chelators, including ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetra acetic acid (EGTA), N,N,N',N'-tetrakis(2-pyridylmethyl) ethylenediamine (TPEN) and bathocuproine enhanced the aqueous dissolution of Aβ from post mortem AD brain tissue. A significant increase in the concentration of Aβ in soluble fractions following chelation treatment was reportedly due to the selective chelation of Cu(II), Zn(II), and Fe(III) ions but not the abundant and essential Ca(II) and Mg(II) ions [9].

We initiated this study firstly to determine if cortical amyloid burden is correlated with neuropathological assessments of amyloid load and secondly to develop a model system for the evaluation of chelators on the resolubilization of Aβ in vivo. We demonstrate that Aβ levels are markedly increased in AD (AD3) brains when compared to controls (AD0). Furthermore, we confirm the effect of TPEN on resolubilizing Aβ in human brain tissue [9], and have extended these findings to show that the lipid soluble chelator and antioxidant, 1,2-dithiolane-3-pentanoic acid (β-lipoate or lipoic acid [36,37]), can resolubilize Aβ from human AD brain.

2. Methods

2.1. Brain tissue selection

Brain tissue samples of 70 autopsy cases were used for this study. Samples were taken as 3 cm × 3 cm × 2 cm blocks, from at least 3 different cortical areas (temporal, frontal (Brodmann's 8,9) and parietal (Brodmann's 39). The blocks taken from the temporal lobe included the hippocampus and part of the entorhinal cortex. Two sets of brain samples from the corresponding regions described above were formalin fixed or frozen in liquid N2 and stored at −80°C prior to processing for biochemical and immunochemical analysis.

AD-type histological changes were assessed in brain tissue samples after formalin fixation (3–4 weeks). Paraffin and frozen sections cut from all these blocks were stained with the Gallyas silver technique, Thioflavin S, and were immunostained with monoclonal antibodies to Aβ protein (DAKO, M 872, dil. 1 : 100) using the avidin-biotin-peroxidase technique.

For the assessment of the degenerative AD-type cortical changes a semi-quantitative analysis and a staging procedure was used, similar to those previously described [29]. The semi-quantitative analysis of senile plaques was made on Thioflavin-S and on Aβ immunostained sections while for neurofibrillary tangles and neuropil threads Thioflavin S and Gallyas silver stained sections were used (Fig. 1). Sections were graded independently by two investigators according to the criteria proposed by Khachaturian [20], CERAD [31] and the NIA-Raegen Institute [2]. After all cortical areas were rated they were correlated and finally staged following Braak et al. [6].

2.2. Transgenic mouse brain

Brain tissue was removed from 14 month old transgenic mice (Tg2576) expressing human mutant AβPP(K670N,M671L) [13,17]. This transgenic mouse line develops extracellular Aβ deposits in the cortex and hippocampus within 9–12 months of age. The presence of elevated Aβ levels in the frontal cortex of these mutant AβPP transgenic mice was determined by western blot analysis with the Aβ-specific monoclonal antibody WO2 (raised against residues 5–8 of the Aβ sequence).

2.3. Preparation of brain homogenates

The frontal cortices from human or murine brains (1g wet weight) were homogenised with a blade homogenizer in Tris-buffered saline (TBS buffer, pH 7.2: 1.5 ml), in the presence of the protease inhibitors: aprotonin (5 μg/ml), phenylmethylsulfonylflouride (PMSF; 0.2 mM) and leupeptin (5 μg/ml). The resulting homogenate (fraction 0) was then fractionated by cen-

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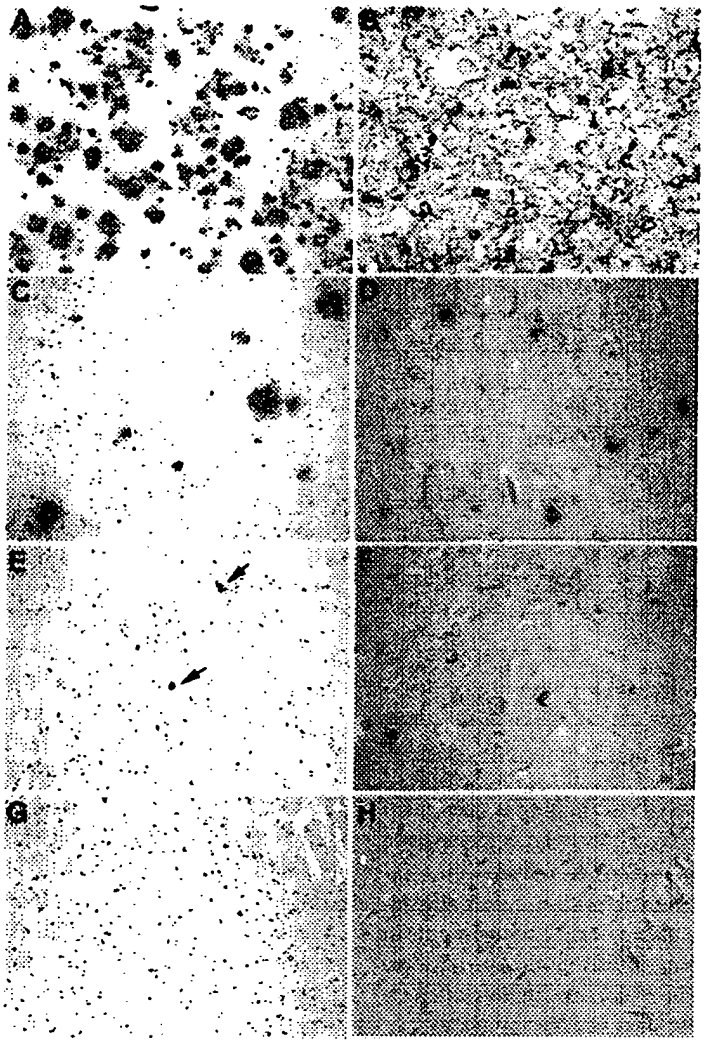

Fig. 1. Morphology of AD type cortical changes: Illustration of the morphological basis of the semiquantitative histological analysis of the AD-type cortical changes used in the 70 human autopsy cases. The photomicrographs A–H were taken from paraffin sections of the frontal associative cortex (Brodmann 8,9). A, C, E and G: amyloid β immunostaining showing different densities of senile plaques. The immunoreaction in A was revealed in black using diaminobenzidin (DAB) and ammonium nickel sulfate, and in C, E and G in brown colour using DAB as chromogen. Photomicrographs in B, D, F and H were taken from Gallyas-stained paraffin sections which were counterstained with light green and show different densities of neurofibrillary tangles. A and B illustrate high density (+++); C and D moderate (++) E and F low density of plaques and tangles, respectively. Note that in E only traces of amyloid-β deposits are seen (arrows). G and H illustrate cases without any plaques or tangles which correspond to grade ( ). This semiquantitative analysis of the AD-type cortical changes was performed in addition to the frontal cortex in the parietal associative cortex and in the temporal cortex. The temporal cortex included the hippocampus and the entorhinal cortex. This enabled us to settle the VI different stages of the AD-related cortical destruction as proposed by Braak. Cases with Braak stages I and II were considered as group AD1 (discrete AD type cortical changes), with Braak stages III, IV as group AD2 (moderate AD-type changes) and those with Braak stages V and VI as group AD3 (severe AD changes). Khachaturian's and CERAD criteria, but also of the NIA-Raegen Institute were all considered in all cases. The group AD3 fit all the criteria for the conventional, definite diagnosis of AD. We considered as controls (AD-) only cases without any plaques or tangles in the cerebral cortex.

trifugation at 10,000 g for 10 min, the 10,000 g pellet fractions (fraction 1) washed six times in homogenisation buffer to liberate residual soluble protein and the 10,000 g supernatant fractions (fraction 2) centrifuged at 160,000 g for 30 min, to produce a high-speed pellet (fraction 3) and supernatant (fraction 4) containing soluble protein.

The protein concentration of each fraction was determined by the Micro Bicinchoninic Acid Assay Kit (Pierce Rockford, IL, USA) using a bovine serum al-

humin standard curve. The A β content of each fraction was determined by Western analysis and quantitated by photodensitometry as described below.

2.4. Formic acid extraction of Aβ from AD brains

To determine the solubility of endogenous Aβ in formic acid, 300 μg of total protein from the initial homogenate (fraction 0) was solubilized in 1 ml of 99% formic acid prior to fractionation. Solubilized Aβ was measured in selected AD3 brain samples. Resulting fractions were air-dried prior to resolubilizing in sample buffer and the Aβ content of 20 μg of total protein from each fraction was determined by Western analysis and quantitation by photodensitometry. Formic acid treated samples were compared to control samples not treated with formic acid.

2.5. Chelation treatment of samples

All chelator preparations were made up in solution with a pH of 6.6. It was critical for such preparations to have identical pH values across different dilutions and with different chelators, as slight deviations either affected the solubility of Aβ directly or the action of chelators (data not shown). This finding is consistent with the work of Atwood et al. [3] who demonstrated that Aβ aggregation by copper was induced by a reduction in pH and was reversible with either alkalinization or chelation. The tendency for Aβ to aggregate in mildly acidic environments and resolubilize in alkaline environments can mask the effect of chelation, or cause false results based on pH rather than treatment.

Washed fraction 1 pellets (500 μg) were incubated in solubilization buffer (100 mM HEPES, 150 mM NaCl, pH 6.6, 50 μl) with and without β-lipoate or TPEN at concentrations of 0.1 mM, 2 mM, and 5 mM at 37°C for 1 h immediately preceding fractionation at 10,000 g for 20 min. The amount of Aβ in the resulting soluble fraction was then determined by standardised Western analysis and photodensitometry analysis described below.

2.6. Immunoblot analysis and photodensitometry

Electrophoresis was performed by a modification of the procedure of Schagger and Von Jagow [44]. A 13% polyacrylamide resolving gel (3 cm × 1.5 mm) was overlayed with a 2.5 cm 8% spacer gel and a 1.5 cm 4% stacking gel. Polyacrylamide gels were prepared in 1 M Tris-HCl (pH 8.45) containing 0.1% SDS. The cathode buffer used was 0.1 M Tris-HCl (pH 8.25), 0.1 M Tricine containing 0.1% SDS, and the anode buffer was 0.1 M Tris-HCl (pH 8.9). The sample buffer contained 83mM Tris-HCl (pH 6.8), 4% SDS, 2% glycine, 6 M urea, 10% β-mercaptoethanol, and phenol red (0.01%). Between 5 μg and 150 μg of total protein from sample fractions were added to sample buffer and heated at 95°C for 5 min. Samples were electrophoresed for 2–2.5 h (50 V through stacking gel and 85 V through spacer and resolving gels). In addition to brain extracts, each gel included Aβ1-40 peptide standards ranging in amount from 10 pg to 600 pg. Resolved proteins were transferred onto a 0.2 μm (Biorad) nitrocellulose membrane at 250 mA overnight at 15°C. Membranes were boiled in PBS for 5 min. immediately following transfer of proteins.

Nitrocellulose membranes were blocked for 45 min. in 2.5% casein to prevent non-specific binding of antibodies. The primary monoclonal antibody, WO2 [gift from Prof. Colin Masters, (University of Melbourne) and Prof. Konrad Beyreuther (Heidelberg University)], which binds residues 5–8 of the Aβ peptide [24] was applied in phosphate buffered saline (PBS, pH 7.4) containing 0.05% Tween 20 for 2 h. Membranes were washed 3 × 10 min. in TBS containing 0.05% Tween 20. The secondary antibody (horseradish peroxidase conjugated anti-mouse IgG – Amersham) was applied at a dilution of 1/10,000 in TBST for 45 min. Membranes were washed as before prior to a 1 min. incubation in Pierce chemiluminescent substrate. All incubations were performed at room temperature with continual rocking. Development of film (Amersham) was performed in accordance with the manufacturer's protocol. Quantitation of bands was performed with photodensitometry using NIH image and a flatbed scanner with transparency attachment.

Means and standard errors were calculated using conventional statistical techniques. Differences between means were tested for statistical significance using one factor ANOVA, or t-test for independent samples with values of $P < 0.05$ considered significant.

3. Results

3.1. Histological analysis

Based on these semi-quantitative histological analyses (see Fig. 1) and staging procedures, the 70 human cases analyzed here were divided into four groups (AD3, AD2, AD1, and AD0). There were 17 cases

with severe AD type changes (AD3, Braak stages V and VI), 20 cases with moderate (AD2, Braak stages III and IV), 19 cases with discrete AD-type changes (AD1; Braak stages I and II) and finally 14 cases without AD-type cortical changes (AD0). In all cases with severe AD-type cortical changes (AD3 group; mean age 75, range 56–90), the histological criteria for the definite diagnosis of AD as proposed by Khachaturian [20], CERAD [31] and following the criteria of the NIA-Raegen Institute [2] were present together with clinical diagnosis of dementia. The severity of the cortical changes corresponded to Braak stages V and VI in these cases, indicating that abundant tangle formation was present not only in the entorhinal cortex and hippocampus but also in the frontal and parietal associative cortex. The cases of the AD3 group fit the conventional criteria for the diagnosis of Alzheimer's disease.

The group AD2 consisted of non demented patients (mean age 80, range 60–90) where the neuropathological examination revealed moderate AD-type cortical changes, insufficient for the neuropathological diagnosis of AD. Despite the high accumulation of senile plaques, neurofibrillary tangles were present in entorhinal cortex and hippocampus but not in associative cortical areas indicating that the severity of the cortical changes corresponded to stages III–IV following Braak. Cases with rare plaques or tangles formed the AD1 group (mean age 78, range 51–92). Only those non-demented cases, where the histological analysis did not show any plaques or tangles were considered as controls and placed in the AD0 group (mean age 67.7, range 42–89).

3.2. Quantitation of Aβ in control and AD brain

The Aβ content from homogenized frontal cortex was analyzed in control brains (AD0) and those with Alzheimer's type cortical changes (groups AD1, AD2 and AD3; Fig. 2(A)). In most of the control subjects Aβ was not detected. A progressive increase in the content of Aβ was observed in brains corresponding to neuropathological grading with AD0 < AD1 < AD2 < AD3 (Fig. 2(B)). However, only AD3 was significantly different from AD0 ($P = 0.003$).

The concentration of Aβ in brain tissue also was analyzed in respect to the semiquantitative analysis of plaque density (Fig. 2(C)). Tissue that did not exhibit any plaques (SP −) comprised 15 AD0 (68%, cases without plaques and tangles) and 7 AD1 (32%, cases without plaques but with few tangles) samples and demonstrated the lowest concentration of Aβ (0.04 ± 0.19 ng/mg) with most of these tissues not containing any detectable levels of Aβ. Due to low sample sizes in tissues exhibiting discrete (SP +) and moderately dense plaques (SP + +), the results of these samples were pooled together. The 11 samples in this group of tissues without severe plaque density were totally comprised of AD1 brain tissue and contained detectable levels of Aβ (3.24 ± 8.09 ng/mg). Tissue with severe plaque density was comprised of all 20 non-demented AD2 (54%) and all 17 demented AD3 (46%) cases. Concentration of Aβ in this group with severe plaque density was the greatest (8.74 ± 13.93 ng/mg; $P < 0.0003$ vs AD0).

3.3. Aβ solubility

The solubility of Aβ in aqueous buffer was investigated in AD3 brains. Homogenate fractions were prepared for each sample as (TBS) or formic acid treated fractions and electrophoresed as described in the materials section. Western blot analysis of Aβ content recovered from each fraction with TBS and formic acid treatment is presented in Fig. 3. Centrifugation at 10,000 g of frontal cortex homogenates in TBS for 20 min. resulted in the formation of a pellet (fraction 1) and supernatant (fraction 2). The majority of Aβ was detected within the pellet fraction (89%). Further fractionation of the 10,000 g supernatant (fraction 2) at 160,000 g for 30 min. resulted in a marked reduction of Aβ in the resulting supernatant (fraction 4) to only 0.3% of total Aβ. The presence of 80% formic acid in homogenates shifted Aβ solubility into the supernatant (fraction 2) after fractionation at 10,000 g. Further fractionation at 160,000 g resulted in only 2% of Aβ being detected in the 160,00 g pellet (fraction 3), demonstrating that the majority of the insoluble Aβ species was formic acid soluble.

3.4. Chelation treatment of samples

Since the solubility studies demonstrated that the majority of Aβ was present in the 10,000 g pellet fraction, we isolated this fraction for treatment with selected chelators. Human AD brains (AD3 frontal cortices) were exposed to 0, 0.1, 2 and 5 mM of TPEN or lipoic acid or a 1 : 1 combination of each compound (Fig. 4(A) and Table 1). TPEN increased the solubility of Aβ in a dose-dependant manner ranging from 0.1 mM to 5 mM. Lipoic acid resolubilization of Aβ was not significantly different from control treatment, although a positive dose response curve was still evident. Combination chelator treatment showed no significant improvement in Aβ solubility when compared to TPEN treatment alone.

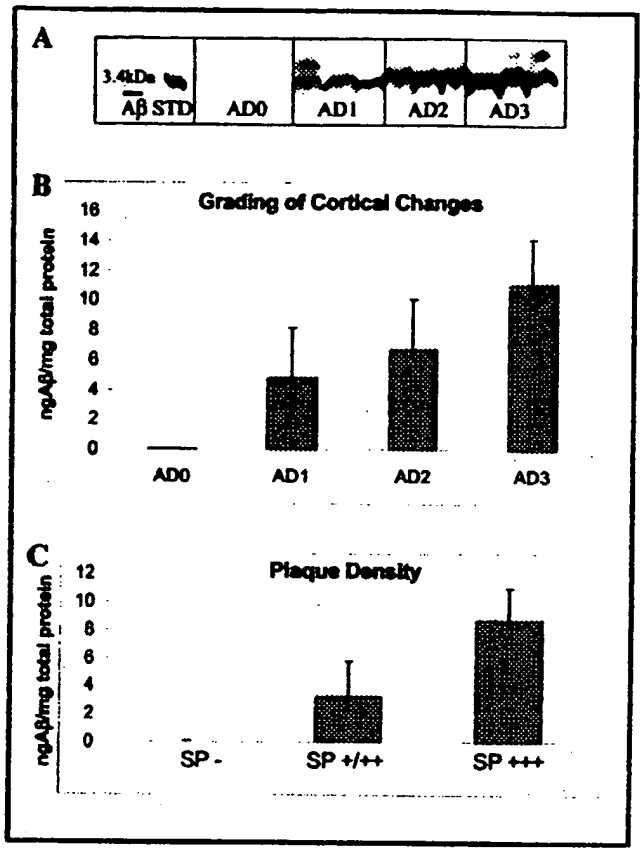

Fig. 2. Aβ detection in control and AD brain: Frontal cortex samples from controls ($n = 14$) and from brains with discrete (AD1; $n = 19$), moderate (AD2; $n = 20$) and severe (AD3; $n = 17$) AD-type cortical changes were homogenised in TBS, pH 7.4. (A) Aβ detected in homogenised samples was visualised by Western Blot analysis using the anti-Aβ monoclonal antibody WO2. (blot shows 3 samples of each AD score representative of mean Aβ detected in 20 μg total protein). Aβ detected in total homogenate was quantified by densitometry of resulting protein bands (Graphs representative of 70 samples). Data presented compares Aβ concentration with the four groups with discrete, moderate, severe and without cortical changes (B) and with the semiquantitative analysis of plaque density (C).

3.5. *Mouse brain*

Frontal cortex tissue from two 14–16 month old Tg2576 transgenic mice was treated with 0, 0.1, 2 and 5 mM TPEN and 0, 0.1, 2 and 5 mM lipoic acid. Western blot analysis for Aβ in 10,000 g supernatant for treated samples from one representative animal is presented in Fig. 4(B). As with human tissue, TPEN enhanced Aβ solubility from the 10,000 g supernatant fraction in a dose-dependant manner, while resolubilization of Aβ with lipoic acid was only detected at the highest concentration of lipoic acid (5 mM).

4. Discussion

Our data demonstrates that Aβ levels are positively correlated with increasing neuropathological severity and clinical signs of dementia. Our results are in agreement with the recently published work of Näslund et al. [34] who demonstrated a correlation between total cortical Aβ concentration and the degree of dementia. Aβ levels generally increased with the severity of cortical changes (Fig. 2(B)), and particularly with the density of plaques (Fig. 2(C)). However, Aβ levels were significantly higher in demented (AD3) individuals ($P = 0.003$) when compared to normal controls, without any plaques or tangles. The increased levels of Aβ in this group (AD3) is consistent with the postulated pathogenic role of this peptide. Dementia was only present in 17 of the 37 cases (46%) with dense amyloid plaques in the frontal cortex. This observation is consistent with the view that while amyloid plaques may reflect the degree of Aβ burden in the AD brain, the clinical status of AD may be better determined by

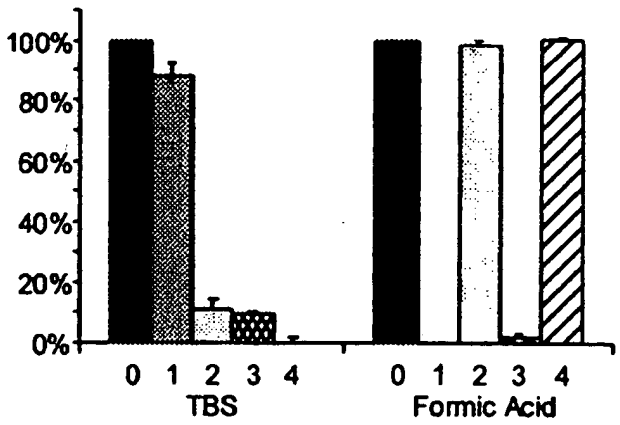

Fig. 3. Solubility of Aβ in 10,000 g and 160,000 g fractions: Data presented as percentage of Aβ?detected in total homogenate (TBS fraction 0). Frontal cortex of 8 neuropathologically confirmed definite AD brains was homogenised in TBS, pH 7.4 and either treated further with TBS or made up to 80% formic acid. Homogenates were fractionated at 10,000 g for 20 min. and the Aβ content of each fraction was determined by quantitation of western blots using anti- Aβ monoclonal antibody WO2. The supernatant fraction (2) was further fractionated at 160,000 g for 30 min. and the Aβ content of resulting fractions (3 & 4) was determined similarly.

Table 1
Aβ resolubilized from insoluble pellet fraction by transition metal chelation

Treatment	n	soluble A-beta (X ± SEM)
HEPES	8	16.4 ± 6.67 pg
		0.24 ± 0.09%
TPEN 0.1 mM	8	20.57 ± 9.08 pg
		0.30 ± 0.12%
TPEN 2 mM	8	36.08 ± 11.14 pg
		0.55 ± 0.15%
TPEN 5 mM	8	57.29 ± 12.39 pg**
		0.91 ± 0.15%**
α-Lipoate 0.1 mM	8	7.71 ± 3.21 pg
		0.11 ± 0.04%
α-Lipoate 2 mM	8	10.16 ± 3.60 pg
		0.15 ± 0.05%
α-Lipoate 5 mM	8	18.53 ± 8.03 pg
		0.27 ± 0.11%
Combination 0.1 mM	7	17.20 ± 6.15 pg
		0.26 ± 0.08%
Combination 2 mM	7	25.98 ± 6.38 pg
		0.42 ± 0.08%
Combination 5 mM	7	47.76 ± 10.24 pg*
		0.77 ± 0.13%*

*$P < 0.05$. **$P < 0.0001$.

the presence of other Aβ species in addition to the deposited species contained within the dense amyloid plaques themselves [28]. These other forms of Aβ may include soluble forms of Aβ, very small deposits not visible by histology, and cerebral amyloid angiopathy (CAA). CAA is present in 83–96% of autopsy confirmed AD [12,41]. Interestingly, and as noted by other workers we noted diffuse plaques and low levels of Aβ in non-demented individuals [14,27,34]. The presence of plaques without dementia has been explained in the context of diffuse plaques having little effect on synapse density and structure with only dense amyloid plaques, seen at the end stages of typical AD, being capable of synapse destruction at the plaque region [21].

While these results suggest that total Aβ level is of greater predictive value of disease status than the presence of dense neuritic plaques per se, these findings do not question the importance of the neuritic plaques in contributing to AD pathogenesis. Indeed, our results indicate that the majority of Aβ (89%) is pelletable at 10,000 g while 11% of Aβ within the Alzheimer's brain exists as either soluble Aβ or very small aggregates not pelleted at 10,000 g (see Fig. 3). The presence of unpelletable Aβ in the AD brain may be explained by the de novo generation of (soluble) Aβ from AβPP that contributes to the soluble Aβ fraction. In addition, it is likely that deposited Aβ may exist in homeostasis with the soluble pool, i.e deposited Aβ may serve as a reservoir of soluble and intermediate aggregates of Aβ.

Formic acid has previously been utilised to completely solubilize Aβ aggregates in vitro [15,26,42,43]

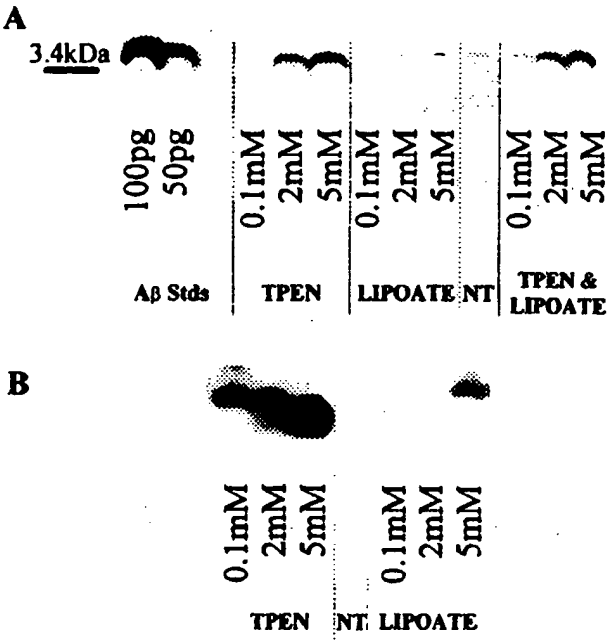

Fig. 4. Chelator mediated extraction of Aβ from AD and Tg2576 brains: Frontal cortex from human brains ($n = 8$) where the diagnosis of definite AD was histologically confirmed and Tg2576 AβPP transgenic mouse brains ($n = 2$) were homogenised in TBS buffer, pH 7.4. Resulting homogenates were fractionated at 10,000 g for 20 min. and the pellet fraction washed 10 times in 100 mM HEPES buffer containing 150 mM NaCl (pH 6.6) with 20 min. centrifugation at 10,000 g between each wash. To 500 μg (total protein) of washed pellet was added 50 μl of 0.1 mM, 2 mM and 5 mM chelator solutions (TPEN, β-Lipoate, a combination each of these preparations or no treatment (NT) prepared in the wash buffer (pH 6.6)). Samples were incubated at 37°C for 1 h and then centrifuged for 20 min at 10,000 g. (A) Aβ content of the resulting supernatant from AD brains was quantitated by Western blot of 30 μl of this supernatant fraction using the monoclonal anti-Aβ antibody WO2. Treatments were assessed by comparing the Aβ content of supernatant fractions to the total Aβ content of starting pellets (Table 1). (B) AβPP transgenic mouse brains were treated with varying concentrations of TPEN and lipoic acid similarly.

and was used in the current study (Fig. 3) to assess dissolution of Aβ aggregates. Our data indicated that the SDS sample buffer (containing 6 M urea and 10% β-mercaptoethanol) employed in this study was equally effective as formic acid in completely solubilizing Aβ aggregates. While these reagents served to demonstrate the maximum solubility of Aβ aggregates they have no therapeutic value.

Treatment of brain tissue with the transition metal ion chelators TPEN and lipoic acid (Table 1, Fig. 4) increased Aβ resolubilization. Thus, our data indicate the possibility that Aβ deposits located within affected areas of the AD brain may be solubilized by treatment with chelators of the transition metal ions that are thought to promote the formation of Aβ aggregation. Our results support those of Cherny et al. [9] who demonstrated significant increases in Aβ solubility in total brain homogenate when chelated with TPEN, EGTA and bathocuproine. However, in contrast to Cherny et al. [9], by removing endogenous soluble Aβ prior to the addition of chelators in our study we avoided the potential complication of varying soluble Aβ levels in AD brain tissues.

In this study higher concentrations of lipoic acid were required for the recovery of Aβ from insoluble amyloid aggregates when compared with TPEN. Although there was no significant change in solubility using lipoic acid, this result may not necessarily preclude lipoic acid from further studies as relatively high doses of this preparation can be maintained without side effects [39]. In support of the value of higher concentrations of lipoic acid, Grunert [16] demonstrated that mercury poisoned mice were protected when treated with a single high dose, but not with a low dose of the agent. The efficacy of treatment may be further enhanced by the employment of multiple doses of lipoic acid rather than a single dose of this compound [11,16]. Thus the efficacy of lipoate, or other potential chelator preparations, in solubilizing Aβ deposits in the brain must await further evaluation by determining optimum dose responses and treatment frequency studies under both in vitro and in vivo conditions.

Although TPEN is of limited benefit due to its highly toxic nature [1] β-lipoic acid is readily absorbed from the diet, and is transported to various tissues, including the brain [38,40] without adverse effects at high concentrations (toxic dose being 400–500 mg/kg of body weight in mice) in-vivo [39]. Therefore, taken together with our in vitro results the chronic ingestion of β-lipoic acid as a therapeutic agent in the prevention or reversal of amyloid plaque formation should be evaluated. AβPP-transgenic animal models provide a useful means of further evaluating chelators as potential therapeutic agents for Alzheimer's Disease.

4.1. Conclusion

In this study we demonstrate that the Aβ load of the frontal cortex is significantly greater in individuals with a definite diagnosis of AD than in control subjects without any AD-type cortical changes. The Aβ concentration correlates with amyloid plaque density and has a predictive value as it is increased in preclinical stages of AD. In addition we have shown that Aβ may be solubilized from Aβ aggregates isolated from AD brains with the use of the transition metal chelators TPEN and β-lipoic acid. Chelators that resolubilize Aβ and promote the increased clearance of soluble Aβ from the brain may be an effective adjunct to AD therapies. We suggest that further studies, including the in-vivo assessment of chelators on Aβ deposition in AβPP transgenic mouse models, are required to further assess the potential of chelators in AD therapy.

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